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ACADEMIC PRESS

Biochemical and Biophysical Research Communications 293 (2002) 396–402

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Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors☆

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Received 18 March 2002

Abstract

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF, identical to prokineticin 1) is a novel peptide recently identified as a selective mitogen for endocrine gland endothelial cells. The present study demonstrates that EG-VEGF/prokineticin 1 and a peptide closely related to EG-VEGF, prokineticin 2, are cognate ligands of two orphan G-protein-coupled receptors designated ZAQ (=EG-VEGF/PK-R1) and I5E (=EG-VEGF/PK-R2). EG-VEGF/prokineticin 1 and prokineticin 2 induced a transient increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$) with nanomolar potency in Chinese hamster ovary (CHO) cells expressing EG-VEGF/PK-R1 and -R2 and bind to these cells with high affinity and with different receptor selectivity. EG-VEGF/prokineticins provoke rapid phosphorylation of p44/42 MAP kinase and DNA synthesis in the bovine adrenal capillary endothelial cells (BACE). The mRNAs of both EG-VEGF/PK-R1 and -R2 were expressed in BACE. The identification of the receptors for EG-VEGF/prokineticins may provide a novel molecular basis for the regulation of angiogenesis in endocrine glands. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: G-protein-coupled receptor; Prokineticin; EG-VEGF; MIT1; GPR73; EG-VEGF/PK-R; Bovine adrenal capillary endothelial cells; MAP kinase; Thymidine incorporation

A wide variety of biologically active substances exert their activity by binding to G-protein-coupled receptors (GPCRs). Recent progress in genome DNA research has identified a large number of genes that encode GPCRs. Many of these are called orphan GPCRs, for which the cognate ligands remain to be unknown. Orphan GPCRs have been used to discover their novel endogenous peptide ligands such as nociceptin/orphanin FQ [1,2], prolactin-releasing peptide [3], orexins [4], apelin [5], ghrelin [6], and metastin [7].

EG-VEGF is a novel angiogenic mitogen that is selective for endocrine gland endothelial cells, and which was recently identified among a library of secreted proteins [8]. EG-VEGF is identical to prokineticin 1 [9], which was recently cloned as a mammalian homolog of mamba intestinal toxin-1 (MIT1) [10]. Prokineticin 1 has a family peptide, prokineticin 2 [9], which is also known as a mammalian homolog of frog skin peptide Bv8 [11]. EG-VEGF/prokineticin 1, prokineticin 2, MIT1, and Bv8 [12] contain 10 cysteine residues in their molecules that are in identical positions, suggesting that these peptides have a common evolutional origin. MIT1 and prokineticins have been

☆ The nucleotide sequence reported in this paper has been submitted to GenBank under Accession Nos. AY089983, AY089984, AY089972, AY089973, AY089974, AY089975, and AY089976.

Abbreviations: GPCR, G-protein-coupled receptor; EG-VEGF, endocrine gland vascular endothelial growth factor; CHO, Chinese hamster ovary; BACE, bovine adrenal capillary endothelial cells; RACE, rapid amplification of cDNA end; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine; FBS, fetal bovine serum; FLIPR, fluorometric imaging plate reader; EC_{50}, half maximum effective concentration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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PII: S0006-291X(02)00239-5

reported to contract gastrointestinal smooth muscles [9,13], although the molecular structure of the receptor remained undefined.

We now report the isolation EG-VEGF/prokineticin 1 from bovine milk and the identification of EG-VEGF/prokineticins as cognate ligands for two closely related orphan GPCRs, ZAQ (=EG-VEGF/PK-R1), and I5E (=EG-VEGF/PK-R2). In addition, we report the tissue distribution of rat EG-VEGF/prokineticins and their receptors' mRNAs and identify the mRNAs of the two receptors in BACE. The identification of EG-VEGF/prokineticin receptors should facilitate the development of novel therapeutics for diseases involving excessive angiogenesis in the endocrine glands.

Materials and methods

Cloning of ZAQ and I5E cDNAs and their expression in CHO cells. Two regions of human ZAQ sequence showing high similarity to human I5E (Patent no. WO9846620) were found in GenBank Database (accession number Z69648 and AQ419390). The 5'- and 3'-ends of the sequence were verified by 5'- and 3'-rapid amplification of cDNA ends (RACE) and full-length human ZAQ cDNA (AY089976) was cloned by PCR with primers 5'-GTCGACATGGAGACCACCATGGGGT TCATGG-3' and 5'-ACTAGTTTATTTTAGTCTGATGCAGTCCA CCTCTTC-3'. Full-length human I5E cDNA was cloned by PCR using gene specific primers. The cDNAs obtained were introduced into an expression plasmid, pAKKO-111H [14] and stably expressed in CHO dhFr-cells by a previously described method [15].

Partial fragment of rat ZAQ cDNA was isolated from rat brain Marathon ready cDNA library (Clontech) by PCR using primers, 5'-GTGGTRCGSCAGCTCTCCTGGGAGCA-3' and 5'-CATGCTG TTGCTCATGGCGATGCACTC-3'. The 5'- and 3'-ends of the fragment were extended by 5'- and 3'-RACE using rat brain Marathon ready cDNA library to obtain the full-length cDNA (AY089974). Rat I5E cDNA clone (AY089975) was obtained from rat brain SuperScript Rat cDNA library (Gibco BRL) using Gene Trapper cDNA Positive Clone Selection System (Life Technologies) with biotinylated probes, 5'-CCT CACCAAYCTGCTYATYGCCAACCTGGCC-3' and 5'-GT GGTR CGSCAGCTCTCCTGGGAGCA-3'.

Partial fragments of bovine ZAQ and I5E cDNAs were obtained from BACE by PCR using degenerate primers, 5'-ATYGTSTGCTG CCCCTTYGAGATG-3' and 5'-TAGTAGAGCTGCTGRTCCACB GRCC-3'. The 5'- and 3'-ends of the fragment were extended by 5'- and 3'-RACE and the full-length coding sequences (AY089972 and AY089973) were obtained by PCR using primers, 5'-GCTCCT CCCGGTTCTTTGAAATC-3' and 5'-TGTAGTCTCTGAATCTCA CTGGTGCC-3' for ZAQ, 5'-GCTGGGTGAGAAGGAATAGGGA-3' and 5'-TGCTCTTTAATCTCGCTGGTGGT-3' for I5E.

Purification of the cognate ligand for ZAQ. Three liters of bovine milk was centrifuged and resulting supernatant was acidified with 1 M acetic acid and centrifuged. The supernatant was mixed with two volumes of acetone for protein-precipitation, after centrifugation to remove the precipitate, the clear supernatant was extracted with diethyl ether. The aqueous phase was evaporated and then loaded onto a C_{18} reversed-phase column (Prep C_{18}, Waters) and eluted with 60% CH_3CN/0.1% TFA. The eluate lyophilized was dissolved in 20 mM $HCOONH_4$ (pH 4)—25% CH_3CN and loaded onto an SP-Sephadex C-25 (Amersham Pharmacia Biotech). The column was successively eluted with 200 mM, 500 mM, and 1000 mM $HCOONH_4$ containing 25% CH_3CN. The 1000-mM eluate was lyophilized and separated by reverse-phase column (TSKgel ODS-80Ts, 4.6 × 250 mm, Tosoh) with a linear gradient of 20–60% CH_3CN in 0.1% TFA for 80 min at 1 ml min^{-1}. The active fractions were further fractionated by reverse-phase column (TSKgel Super-Phenyl, 4.6 × 100 mm, Tosoh) with a linear gradient of 15–40% CH_3CN in 0.1% TFA for 75 min at 1 ml min^{-1} and finally purified by reverse-phase column (μRPC C2/C18 ST 4.6/100, 4.6 × 100 mm, Amersham Pharmacia Biotech) with a linear gradient of 35–50% CH_3CN in 0.1% heptafluorobutyric acid for 60 min at 1 ml min^{-1}. The amino acid sequence of the peptide purified was analyzed with a protein sequencer (PE Biosystems Procise 491cLC).

Calcium-mobilization assay using a fluorometric imaging plate reader (FLIPR). The receptor-expressing cells were seeded (30,000 cells/well) into 96-well black-wall microplates 16–24 h before assay. The cells were incubated for 1 h at 37 °C with 4 μM Fluo-3 AM (Dojindo) in H/HBSS (Hanks' balanced salts solutions supplemented with 20 mM HEPES, pH 7.4) containing 2.5 mM probenecid and 0.1% fetal bovine serum (FBS), and then washed four times with the assay buffer. Changes in $[Ca^{2+}]_i$ were measured using a FLIPR (Molecular Devices).

Purification of MIT1 from black mamba venom. MIT1 was purified from black mamba venom (Sigma) monitoring agonist activity for ZAQ using a FLIPR. The venom was fractionated by reverse-phase column (Wakosil-II 5C18HG Prep, 20 × 250 mm, Wako) with a linear gradient of 20–40% CH_3CN in 0.1% TFA for 120 min at 5 ml min^{-1}. The active fractions were lyophilized and then purified by cation exchange column (TSKgel CM-2SW, 4.6 × 250 mm, Tosoh) with a linear gradient of 10–1000 mM $HCOONH_4$ (pH 6.6) containing 25% CH_3CN for 90 min at 1 ml min^{-1}. The peptide was finally purified by reverse-phase column (Vydac 218 TP510, 4.6 × 100 mm, Vydac) with a linear gradient of 1535% CH_3CN in 0.1% TFA for 75 min at 1 ml min^{-1}.

Cloning of EG-VEGF/prokineticin 1 and prokineticin 2 cDNAs. Partial fragment of rat EG-VEGF/prokineticin 1 cDNA was cloned from rat brain cDNA library by degenerate PCR with primers, 5'-TCA CCYCAAGTGAYCATGAGAGG-3' and 5'-CTAAAARTTGRYRT TCTTCAAGTCC-3' for the first PCR, and 5'-ATCACAGGGGC CTGTGARCG-3' and 5'-AGCAGCGGTACCTGCCGTCC-3' for nested PCR. After 5'- and 3'-RACE for extending the external sequences, the full-length cDNA of rat EG-VEGF/prokineticin 1 (AF089983) was isolated by nested PCR with primers 5'-GATCATG AGAGGTGCTGTGCAAGTCTTC-3' and 5'-CAGATGTAACACA AGAGGTCACCCAGTAGG-3' following the first PCR with primers 5'-ATTCCAGAGTGGACAGTGTTTGCCTTCACC-3' and 5'-CTC TCTGCACGCTGCTGGACTGTTCC-3'. Partial fragment of rat prokineticin 2 cDNA was obtained by degenerate PCR using rat testis Marathon ready cDNA library and following primers: 5'-GCTTGYG ACAAGGACTCYCA-3' and 5'-GTTYCTACTYCAGAGYGAT-3'. After 5'- and 3'-RACE for extending the external sequence, the full-length cDNA of rat prokineticin 2 (AY089984) was isolated from rat brain cDNA by nested PCR with primers, 5'-GGGACGCCATGG AGGAC-3' and 5'-TTTCCAGCTCCTGCTTCAGA-3' following the first PCR with primers 5'-TAACCGCCACCGCCTCCT-3' and 5'-CG AGACTTGACAGACATTGTTCAGTG-3'.

Expression of human EG-VEGF/prokineticin 1 and prokineticin 2 in Escherichia coli. The double-stranded DNA encoding human EG-VEGF/prokineticin 1 or prokineticin 2 was obtained by annealing six synthetic oligonucleotides, and inserted into the *NdeI–BamHI* cloning site of the pTCII vector [16]. The expression plasmid was introduced into *E. coli* MM294 (DE3) and recombinant human EG-VEGF/prokineticin 1 and prokineticin 2 were produced under the control of T7 promoter. Recombinant EG-VEGF/prokineticin 1 or prokineticin 2 was extracted from *E. coli* cells with extraction buffer (7 M guanidine–HCl, 200 mM Tris, pH 8.0), refolded with refolding buffer (50 mM Tris, 0.4 M L-arginine, 1 mM reduced glutathione, and 0.2 mM oxidized glutathione, pH 8.5), and purified using a TSKgel CM-5PW column (21.5 × 150 mm, Tosoh) and C4P-50 column (21.5 × 300 mm, Showa Denko). The purified peptide was proved to be homogeneous on SDS–PAGE and analytical HPLC.

Receptor binding assay. The assay was performed by a previously described method [15] with minor modification. MIT1 was radiolabeled with [^{125}I]Bolton-Hunter reagent (NEX120, NEN) and the peptide radiolabeled ([^{125}I]BH-MIT1) was purified using reverse-phase column (TSKgel Super-ODS, 4.6 × 100 mm, Tosoh). The cells were inoculated into 24-well plates and cultured for 2 days. The cells were washed with assay buffer (H/HBSS containing 0.2% bovine serum albumin (BSA)) and were incubated with 100 pM [^{125}I]BH-MIT1 and peptide samples for 37 °C for 1 h. The cells were then washed with assay buffer and then solubilized with 0.5 N NaOH/0.1% SDS. Radioactivity was measured with a γ-ray counter. Nonspecific binding was determined in the presence of 1 μM unlabeled MIT1.

Detection of p44/42 MAP kinase activation. BACE were obtained as described by Folkman et al. [17]. BACE were plated into 12-well plates and cultured for 1 day. After BACE were treated with peptide samples for 5 min at 37 °C, the supernatant was removed and the cells were lysed with lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 50 mM NaF, 4 mM $Na_4P_2O_7$, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 10 μg/ml pepstatin A, pH 7.4). The lysates were centrifuged and the supernatants were subjected to Western blot analysis using Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) Antibody (Cell Signaling Technology) and ECL-plus detection system (Amersham Pharmacia Biotech).

Measurement of [^{3}H]thymidine incorporation into BACE. BACE were plated into 48-well plates and cultured for 1 day. After the cells were cultured with ligands in DMEM containing 0.1% FBS and 0.5% BSA for 16–20 h, the cells were labeled with [^{3}H]thymidine (0.5 μCi/well) for another 8 h. The cells were then washed with cold H/HBSS and methanol and incubated with 10% trichloroacetic acid at 4 °C for 15 min. The acid-insoluble fraction was dissolved with 0.3 N NaOH and the radioactivity was counted using a scintillation counter.

Real time PCR analys is for EG-VEGFlprokineticins and EG-VEGFl PKRs mRNAs. Total RNAs were prepared from multiple tissues of 7–8 week-old Wistar rats using Trizol reagent (Gibco) and poly(A)$^+$ RNAs were purified using mRNA purification kit (Amersham Pharmacia Biotech). cDNAs were synthesized using SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technologies). Real time PCR analysis was carried out using a Prism 7700 Sequence Detector (PE Biosystems). For BACE, total RNA was prepared using RNeasy Mini kit (Qiagen) and was directly subjected to cDNA synthesis and real time PCR analysis. Specific primers and fluorescence-labeled probes used were: 5′-TTGCGAAGCTTTTTGTAGCG-3′, 5′-CAACTTCATCTTCATCACTGCGCTGGC-3′, and 5′-Fam-CAACTTCATCTTCATCACTGCGCTGGC-3′ for rat EG-VEGF/PK-R1; 5′-CACAGACCTTCTTTGCAGCCA-3′, 5′-CAGACTAGCATGATGCC-3′, and 5′-Fam-AATCGTCATTGGCGTAGCCCTGGC-Tamra-3′ for rat EG-VEGF/PK-R2; 5′-GTGCAAGTCTTCATCATGCTCCT-3′, 5′-AGGGGCCTGTGAACGAGAT-3′, and 5′-Fam-CTAGCAACTGTCTCTGACTGTGCGGTGATC-Tamra-3′ for rat EG-VEGF/prokineticin 1; 5′-CCCCCTGACTCGGAAAGTTC-3′, 5′-CCAGGTTTGGCATGTTTAAGG-3′, and 5′-Fam-AGGATGCACCACACTTGTCCCTGCCT-Tamra-3′ for rat prokineticin 2; AACCAACTATTTCCCTCTGCTTGAC-3′, 5′-TCACCGTAGCTGAAGTTGAAGG-3′, and Fam-CCTCGGAGCCCAAGCTGCTTCTTT-Tamra-3′ for bovine EG-VEGF/PK-R1; or 5′-TCATGAGAGCAGAAGGTCTGGA-3′, 5′-TGGCTGGAAAACTAGCATTTCC-3′, and 5′-Fam-CACACACCGCTCACTGGAAAGCTTCA-Tamra-3′ for bovine EG-VEGF/PK-R2.

Results and discussion

During our attempts to search human genomic DNA database for novel GPCR genes, we found two DNA fragments of a putative GPCR gene. Based on these sequences, we isolated the full-length cDNA encoding an orphan GPCR originally termed ZAQ. ZAQ showed significant homology to mouse GPR73 [18] and human orphan GPCR, I5E (Patent no. WO9846620, 90% identity, Fig. 1). It is thus suggested that ZAQ and I5E are the receptor subtypes for structurally related ligands. We subsequently isolated the rat and bovine ZAQ and I5E cDNAs. The deduced amino acid sequences of these proteins are shown in Fig. 1.

To identify the unknown cognate ligand for ZAQ, we monitored the agonist activity of the increase in $[Ca^{2+}]_i$ in CHO cells stably expressing ZAQ using a FLIPR. ZAQ is structurally similar to neuropeptide Y receptors, but did not respond to known neuropeptides, including neuropeptide Y, peptide YY, and pancreatic polypeptide up to a concentration of 1 μM. During screening against several kinds of tissue extracts, we found that bovine

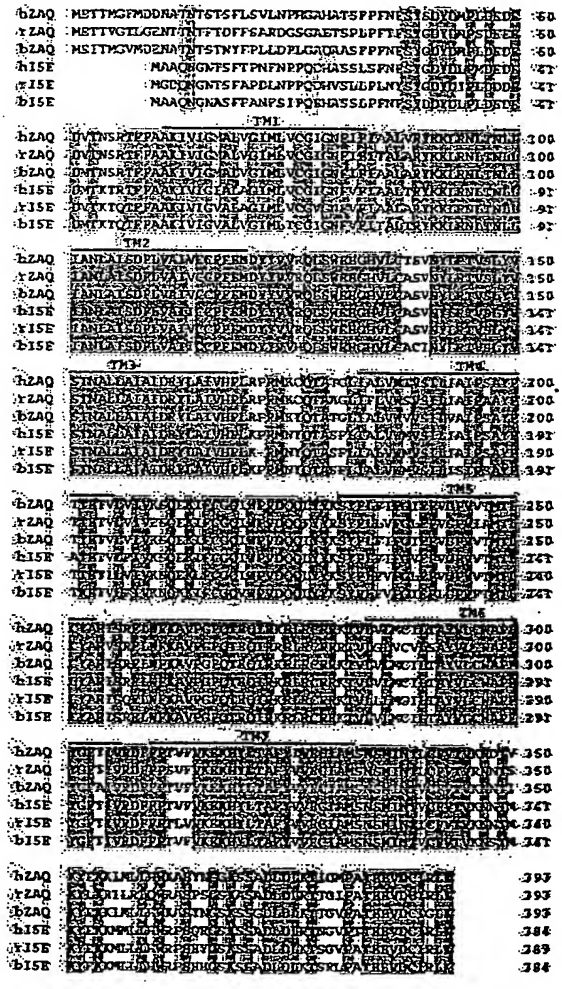

Fig. 1. Alignment of human, rat, and bovine ZAQ (EG-VEGF/PK-R1) and I5E (EG-VEGF/PK-R2) amino acid sequences. Shading denotes amino acid identity. Seven putative transmembrane domains (TM1?) are indicated.

milk exhibited a strong activity that induced a robust and transient increase in $[Ca^{2+}]_i$ (Fig. 2A). This activity was specific for ZAQ, since it failed to evoke an increase in

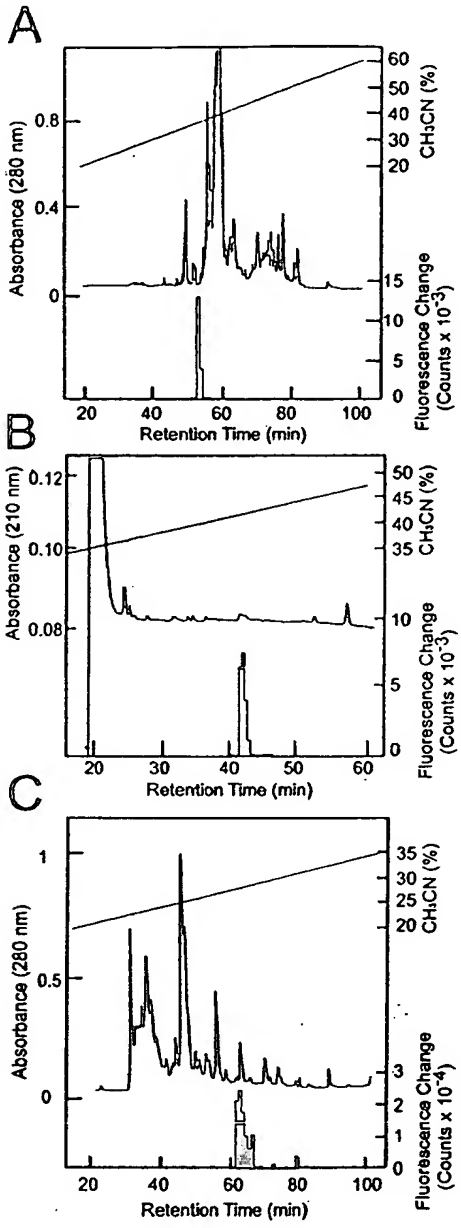

Fig. 2. Purification of EG-VEGF/prokineticin 1 from bovine milk and identification of MIT1 in black mamba venom. (A) The first HPLC purification step of EG-VEGF/prokineticin 1 from bovine milk by reverse-phase column (TSKgel ODS-80Ts). The specific fluorescence changes in EG-VEGF/PK-R1-expressing CHO cells were shown with black bars. (B) The final purification step of EG-VEGF/prokineticin 1 by reverse-phase column (μRPC C2/C18 ST 4.6/100). Active substance, corresponding to the absorbance peak, was subjected to a protein sequencer. (C) Identification of EG-VEGF/PK-R1-activating activity in black mamba venom. The venom was fractionated using reverse-phase column (Wakosil-II 5C18HG). Bars show specific fluorescence change in CHO cells expressing EG-VEGF/PK-R1.

$[Ca^{2+}]_i$ in control of CHO cells expressing ET_A receptor [15]. We purified the activity by successive steps of HPLC from bovine milk (Fig. 2B). The partial N-terminal amino acid sequence of the peptide isolated was determined to be AVITGAXERDVQXRAGTXXAVSL (X, not identified). This sequence was remarkably similar to the N-terminal amino acid sequence of MIT1 [10] isolated from the black mamba (*Dendroaspis polylepis*) venom. A database search revealed the presence of a human expressed sequence tag (X40467). Since the clone was found to be partial, we performed a 3′RACE reaction with human testis cDNA as a template to obtain the full-length cDNA. After the completion of the cDNA cloning, the same sequence was reported as EG-VEGF [8] and prokineticin 1 [9] by two groups. EG-VEGF was identified among a library of human secreted proteins as a mitogen selective for endothelial cells derived from endocrine glands. Prokineticin 1 was cloned as a mammalian homolog of MIT1 with potent contractile activities for gastrointestinal smooth muscles. EG-VEGF/prokineticin 1 shares 80% identity with MIT1 and 58% identity with prokineticin 2 [9] (Fig. 3). Prokineticin 2 was also recently cloned as a mammalian homolog of the frog skin peptide, Bv8 [11]. We further isolated rat EG-VEGF/prokineticin 1 and prokineticin 2 cDNAs and found that these peptides are highly conserved between human and rat (93% and 95% similarity in mature form, respectively) (Fig. 3).

To confirm whether the MIT1-like molecule is cognate ligand for ZAQ, we examined the ZAQ-agonist activity of MIT1 and of recombinant EG-VEGF/prokineticin 1 and prokineticin 2. First, we examined the activity in black mamba venom and found that it showed a specific and an extremely strong activity (Fig. 2C). We then analyzed the purified active substance by mass spectrometry, sequenced it by Edman degradation,

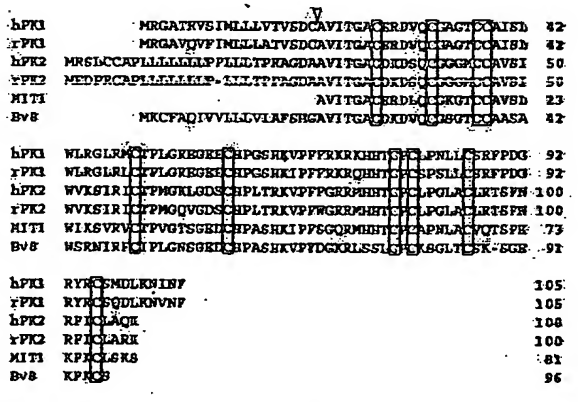

Fig. 3. Alignment of amino acid sequences of rat and human EG-VEGF/prokineticin 1 (PK1), prokineticin 2 (PK2), MIT1, and Bv8. Conservative cysteine residues are shown in boxes. Arrowhead indicates the cleavage site of signal peptide.

and identified it as MIT1 (C-terminal serine residue deleted).

We next produced recombinant human EG-VEGF/prokineticin 1 and prokineticin 2 in *E. coli* and CHO cells. We examined the agonist activity of the peptides for ZAQ and the presumed ZAQ subtype, I5E, using a FLIPR. Since the recombinant EG-VEGF/prokineticin 1 and prokineticin 2 produced in *E. coli* and CHO cells showed comparable agonist activity for the two receptors, we used the recombinant peptides produced in *E. coli* in the following experiments. EG-VEGF/prokineticin 1 and prokineticin 2 induced a dose-dependent transient increase in $[Ca^{2+}]_i$ in ZAQ- and I5E-expressing CHO cells (Fig. 4A and B). The half maximum effective concentration (EC_{50}) of EG-VEGF/prokineticin 1 and prokineticin 2 was 140 ± 29 and 15 ± 2 pM, for ZAQ and 2900 ± 530 and 150 ± 31 pM for I5E, respectively ($n = 5$). These values are low enough to make EG-VEGF/prokineticin 1 and prokineticin 2 good candidates for cognate ligands for ZAQ and I5E. It is interesting that non-mammalian homolog of EG-VEGF/prokineticins, MIT1, showed the most potent agonist activity for ZAQ and I5E with EC_{50} values of 13 ± 2.1 and 34 ± 11 pM, respectively ($n = 5$) (Fig. 4A and B).

Radioligand-binding studies were then performed to further characterize these ligands/receptors pairing. The binding of [^{125}I]BH-MIT1 to ZAQ- and I5E-expressing CHO cells was inhibited by a nanomolar concentration of unlabeled prokineticins and MIT1 in a dose-dependent manner (Fig. 4C and D). The concentration of unlabeled ligands to inhibit 50% of specific radioligand binding (IC_{50}) for EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 was 250 ± 31, 6.9 ± 1.2, and 4.1 ± 0.52 nM for ZAQ, 81 ± 7.4, 7.6 ± 0.90, and 0.67 ± 0.21 nM for I5E, respectively ($n = 4$). These results from the functional and binding assay confirm that EG-VEGF/prokineticin 1 and prokineticin 2 are cognate ligands for both ZAQ and I5E, which we now designate EG-VEGF/PK-R1 and EG-VEGF/PK-R2, respectively. It was shown that EG-VEGF/prokineticin 1 and prokineticin 2 shared EG-VEGF/PK-R1 and -R2, although the two receptors showed different ligand selectivity. EG-VEGF/PK-R1 is a MIT1- and prokineticin 2-preferable receptor, while EG-VEGF/PK-R2 is a MIT1-selective receptor.

Since it was demonstrated that EG-VEGF shows mitogenic activity for BACE [8], we examined the effects of prokineticin 2 and MIT1 on mitogenic parameters for BACE. First, we determined whether EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 activated p44/42 MAP kinase (MAPK) in BACE. Several lines of evidence have shown that MAPK are important integrators for cell proliferation [19]. Activation of p44/42 MAPK was detected by Western blot analysis with Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) Antibody that specifically recognizes the activated form of p44/42 MAPK. EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 stimulated dose-dependent phosphorylation of p44/42 MAPK with a rank order of potency as follows: MIT1 > prokineticin 2 > EG-VEGF/prokineticin 1 (Fig. 5A). Furthermore these three peptides promoted [^{3}H]thymidine incorporation into DNA with similar ligand selectivity (Fig. 5B). The EC_{50} values of EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 for this activity were 1400 ± 240, 66 ± 14, and 22 ± 5.9 pM, respectively ($n = 5$). These three peptides showed strong similarity in both structure and mitogenic activity for capillary endothelial cells derived from the endocrine gland.

To investigate the putative physiological roles of the novel ligands/receptors family, we examined the tissue distribution of EG-VEGF/prokineticin 1 and prokineticin 2 and their receptors in rat using a real time PCR analysis (Fig. 6A and B). The expression of EG-VEGF/prokineticin 1 was generally lower than that of prokineticin 2 in the tissues examined. The expression of prokineticin 2 was highest in the testis. Moderate level of expression was seen in the cerebrum, thymus, lung, spleen, ovary, and skeletal muscle.

To compare the localization of the two peptides with their receptors, we also examined the expression patterns of EG-VEGF/PK-R1 and -R2 in the same set of rat tissues. The result showed distinct expression patterns for the two receptors. EG-VEGF/PK-R1 mRNA was widely distributed in peripheral tissues with the highest level in the spleen and moderate levels in the

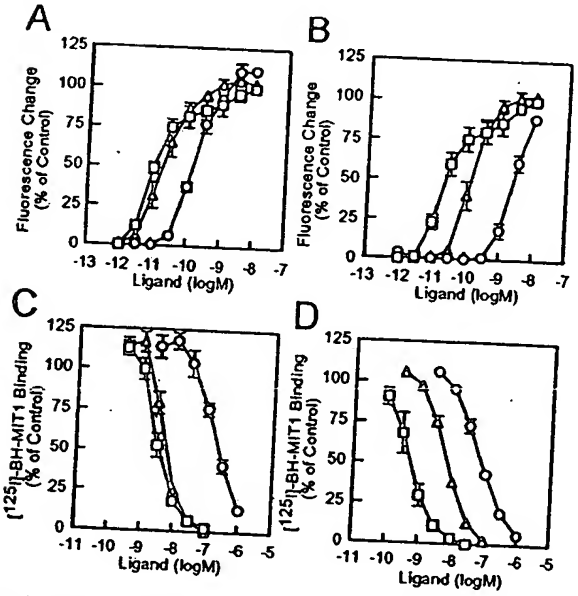

Fig. 4. Pharmacological characterization of EG-VEGF/PK-Rs. (A,B) Dose–response relationship of the increase in $[Ca^{2+}]_i$ by EG-VEGF/prokineticin 1 (circle), prokineticin 2 (triangle), and MIT1 (square) in CHO cells expressing EG-VEGF/PK-R1 (A) or -R2 (B). (C,D) Competitive radioligand binding assays with CHO cells expressing EG-VEGF/PK-R1 (C) or -R2 (D). Symbols used are the same as in A and B. Data are means ± SE of 4–5 individual experiments.

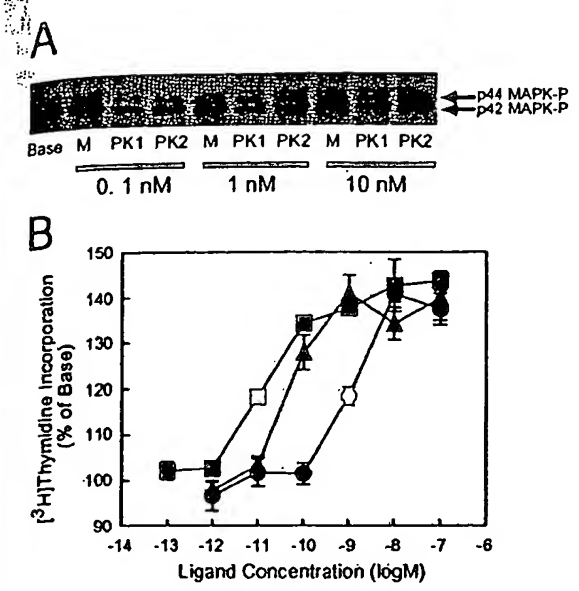

Fig. 5. Effects of EG-VEGF/prokineticin 1, prokineticin 2, and MIT1 on p44/42 MAPK phosphorylation and DNA synthesis in BACE. (A) phosphorylation of p44/42 MAPKs induced by indicated concentration of EG-VEGF/prokineticin 1 (PK1), prokineticin 2 (PK2), and MIT1 (M) was detected with Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) Antibody. (B) [^{3}H]thymidine incorporation induced by EG-VEGF/prokineticin 1 (circle), prokineticin 2 (triangle), and MIT1 (square). Data are mean ± SE of five individual experiments.

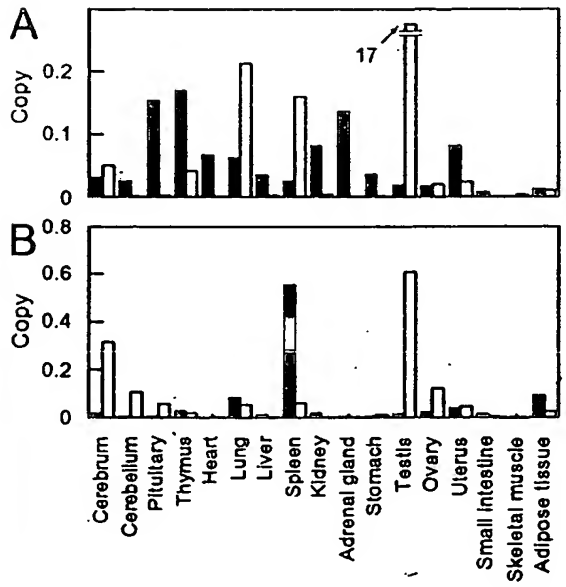

Fig. 6. Tissue distributions of EG-VEGF/prokineticins and their receptors mRNAs in rats analyzed quantitative RT-PCR analyses. (A) EG-VEGF/prokineticin 1, black bars; prokineticin 2, white bars. (B) EG-VEGF/PK-R1, black bars; EG-VEGF/PK-R2, white bars. Values are expressed as ratio to GAPDH × 100 for prokineticin 2, EG-VEGF/PK-R1 and -R2, and as ratio to GAPDH × 1,000 for EG-VEGF/prokineticin 1.

adipose tissues, thymus, lung, kidney, testis, uterus, and small intestine. In contrast, EG-VEGF/PK-R2 mRNA was expressed abundantly in the central nervous system and reproductive organs with the highest levels in the cerebrum, cerebellum, testis, and ovary. To examine the receptor subtype expressed in BACE, we cloned bovine EG-VEGF/PK-R1 and -R2 and quantified the mRNAs of the two receptors. The mRNAs of both EG-VEGF/PK-R1 and -R2 were expressed in BACE (82 copies/ng total RNA for EG-VEGF/PK-R1 and 77 copies/ng total RNA for EG-VEGF/PK-R2), indicating that the mitogenic activity of EG-VEGF/prokineticin 1 and prokineticin 2 is mediated by EG-VEGF/PK-R1 and/or EG-VEGF/PK-R2. Further studies are required to examine which receptor subtype is primarily involved in EG-VEGF/prokineticins-induced proliferation in BACE.

LeCouter and colleagues [8] showed that gene transfer of EG-VEGF in the rat ovary using adenovirus vector resulted in cysts formation with excessive angiogenesis. Future studies are required to examine the involvement of the EG-VEGF/prokineticin system in human ovarian disorders characterized by excessive angiogenesis, such as the polycystic ovary syndrome and ovarian cancer. The identification of EG-VEGF/prokineticins receptors in the ovarian capillary endothelial cells and elucidating the mechanism of EG-VEGF/prokineticins production in the ovary will be essential steps to clarify the pathological role of the EG-VEGF/prokineticins system in such human ovarian disorders. Furthermore, the wide tissue distributions of EG-VEGF/prokineticins and their receptors suggest that the EG-VEGF/prokineticin system, which plays a role in angiogenesis in endocrine glands and gastrointestinal motility, may also be involved in a number of additional, but as yet undefined, physiological roles.

In conclusion, we have demonstrated that EG-VEGF/prokineticin 1 and prokineticin 2 bind to and activate two structurally related GPCRs, EG-VEGF/PK-R1, and -R2. We have also shown the presence of both EG-VEGF/PK-R1 and -R2 mRNAs in BACE. The identification and characterization of these two distinct EG-VEGF/prokineticin receptors will facilitate the understanding of the physiological and/or pathological significance of EG-VEGF/prokineticin and may also lead to the development of a receptor antagonist that could be used to treat endocrine disorders involving excessive hypervascularity.

Note added in proof. During the preparation of the manuscript, a part of the study, the identification of human prokineticins/EG-VEGF receptors was reported [20].

Acknowledgments

We thank Y. Matsumoto and H. Yoshida for helpful discussions.

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0026-895X/01/5904-692-698$3.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 59:692–698, 2001

Vol. 59, No. 4
657/891370
Printed in U.S.A.

Identification of Two Prokineticin cDNAs: Recombinant Proteins Potently Contract Gastrointestinal Smooth Muscle

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Received October 23, 2000; accepted December 26, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The motility of gastrointestinal tract is regulated by classical neurotransmitters, neuropeptides, and humoral agents. Two novel human cDNAs have been cloned based on their sequence similarity to a frog skin secretion protein, Bv8, and a nontoxic protein of mamba snake venom. These human cDNAs encode two secreted proteins of 86 and 81 amino acids. Northern blot hybridization has revealed that these cDNAs are expressed in gastrointestinal tract, particularly the stomach. Recombinant proteins with authentic N-terminal sequences have been produced in *Escherichia coli* and refolded into functional proteins by careful control of protein aggregation. Mass spectrometry has confirmed the formation of five pairs of disulfide bonds. The refolded recombinant proteins potently contract gastrointestinal smooth muscle with EC_{50} values in the subnanomolar range. The contractile effects of the recombinant proteins are specific for gastrointestinal smooth muscle, because they have no effect on vascular or respiratory smooth muscle. To reflect their potent and specific effects on gastrointestinal smooth muscle cells, we have named these recombinant proteins prokineticins. Ligand binding studies with iodinated prokineticin revealed the presence of a high-affinity site in ileal smooth muscle. The displacement of specific binding by GTPγS suggests that the prokineticin receptor may belong to the family of G protein-coupled receptors. Experiments with verapamil and nifedipine revealed that calcium influx is essential for the contractile activity of prokineticins on gastrointestinal smooth muscle. In summary, we have identified two novel endogenous regulators of gastrointestinal motility. The availability of recombinant prokineticins should provide novel therapeutic agents for disorders involving impaired gastrointestinal motility.

The function of gastrointestinal (GI) smooth muscle is to mix and propel intraluminal contents, enabling the efficient digestion of food, the progressive absorption of nutrients, and eventual evacuation of residual components. The activity of GI smooth muscle is regulated by intrinsic and extrinsic neural signals, including classical neurotransmitters, coexisting neuropeptides, and circulating peptide hormones (Fox-Threlked, 1993; Wood, 1994). Also, a number of locally produced humoral agents, including histamine, serotonin, and adenosine, influence the activity of smooth muscle cells (Burks, 1994). In addition to these endogenous agents, some exogenous peptides with contractile activity have been identified. Schweitz et al. (1990; 1999) purified a small protein from mamba venom [mamba intestinal toxin (MIT) 1] and showed that it potently stimulates contraction of the guinea pig ileum. Recently, a protein of similar size with greater than 40% identity with MIT1, including all 10 conserved cysteines, has been purified from frog skin secretion (Mollay et al., 1999). The frog protein, named Bv8, was also found to stimulate the contraction of GI smooth muscle with high potency (Mollay et al., 1999). Because a number of bioactive mammalian peptides or proteins, including bombesin, endothelin, natriuretic peptide, and the secreted form of PLA2, have found their counterparts in snake venom and frog skin secretion (McDonald et al., 1979; Brown et al., 1980; Takasaki et al., 1988; Yanagisawa et al., 1988; Schweitz et al., 1992; Tischfield, 1997), we sought to identify mammalian homolog(s) of frog Bv8 and snake MIT1 that may regulate the GI contractility.

Here, we describe the isolation and characterization of two human cDNAs that encode the homologs for snake MIT1 and frog Bv8. Refolded recombinant proteins were found to stimulate the contraction of gastrointestinal smooth muscle with high potency. We have named these proteins prokineticins to reflect their specific and potent contractile activity on GI smooth muscle. Evidence that prokineticins may interact with a G protein-coupled receptor family is also presented. The discovery of endogenous regulators of gastrointestinal motility should facilitate the development of novel therapeutics for disorders that involve impaired gastrointestinal motility.

ABBREVIATIONS: GI, gastrointestinal; MIT, mamba intestinal toxin; GST, glutathione-*S*-transferase; HPLC, high-performance liquid chromatography; RP, reverse(d) phase; EST, expressed sequence tag.

Materials and Methods

RNA Blot. Human multiple tissue RNA blots containing normalized samples of poly(A) RNA were used according to the manufacturer's instructions (CLONTECH, Palo Alto, CA). The blots were probed with random primer-labeled probes (nucleotides 1–550 and 1–1178 for prokineticin 1 and prokineticin 2 cDNAs), and signals were visualized by exposing to Kodak XAR film.

Cloning of Full-Length cDNAs. For cloning full-length prokineticin 2 cDNA, a 5′ RACE with human brain cDNA mixture (CLONTECH) was performed. The polymerase chain reaction conditions were 94°C for 30 s and 68°C for 2 min (30 cycles). The specific oligonucleotides used were RACE1: ACATGGGCAAGTGTGATGCAT and RACE2: ATTACTTTTGGGCTAAAC.

Production, Refolding, and Purification of Recombinant Prokineticins. The coding sequences for mature prokineticins were cloned into the prokaryotic expression vector pGEX-3X (Amersham Pharmacia Biotech, Piscataway, NJ). The extra nucleotides between the factor Xa protease digestion site of the glutathione-*S*-transferase (GST) tag and mature prokineticins were removed by site-directed mutagenesis and confirmed by sequencing. To facilitate protein purification, a 6xHis-tag was added to the C terminus so that the fusion proteins could be purified with Ni-NTA affinity chromatography (Qiagen, Valencia, CA). The detailed protocols for production of fusion proteins are as follows. *Escherichia coli* cells (BL21) were grown to absorbance 0.8 and induced with 600 μM isopropyl β-D-thiogalactoside for 2 h at 37°C. The cells were then pelleted, washed, and lysed with buffer A (6 M guanidine hydrochloride, 100 mM NaH_2PO_4, and 10 mM Tris, pH 8.0). Fusion proteins were allowed to bind to Ni-NTA beads and then washed extensively with buffer C (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 6.3) and buffer D (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 5.9). Fusion protein-bound beads were equilibrated with digestion buffer (50 mM Tris, 150 mM NaCl, and 1 mM $CaCl_2$, pH 7.5). Digestion was performed overnight at room temperature with 10 ng of protease factor Xa per microgram of fusion protein. The cleaved GST tag was then washed away with buffer D. Mature prokineticins were eluted with buffer E (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 4.5), and fractions were analyzed by SDS-PAGE. The pooled recombinant prokineticins were then refolded as follows. Proteins were diluted to 100 μg/ml with buffer E and dialyzed against renaturing buffer (4 M urea, 5 mM cysteine, 0.02% Tween-20, 10% glycerol, 10 mM Tris, 150 mM NaCl, 100 mM NaH_2PO_4, pH 8.3). New renaturing buffer (same as above except with 2 M urea) was then added, and dialysis was continued for 4 more days with at least one more change of renaturing buffer. The refolded protein was then desalted with a spin column (Qiagen) and analyzed by receptor binding or bioassay. The final purification was performed with reverse phase-HPLC (Amersham Pharmacia Biotech). Functional proteins were eluted with 0.08% trifluoroacetic acid and a 10 to 50% acetonitrile gradient. The elution of protein was monitored at 206 nm. Trifluoroacetic acid and acetonitrile were then evaporated by lyophilization.

Mass Spectrometry. The electrospray ionization mass spectrometry was performed with a 6.5-T HiResESI Fourier Transform mass spectrometer (IonSpec, Irvine, CA) as described previously (Li et al., 1994) with a sample volume of 100 μl. Protein eluted from RP-HPLC was lyophilized and dissolved in nanopure water and then diluted to a concentration of 1 μM with methanol/water/acetic acid (49.5%:49.5%:1%, v/v/v).

Isolated Smooth Muscle Preparations. Guinea pigs were euthanized with CO_2, and a section of ileum (2–3 cm) approximately 10 cm rostral to the cecum was removed. The tissue was washed clean with Krebs-Ringer-bicarbonate buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, and 10 mM glucose) and mounted longitudinally in an organ bath containing Krebs-Ringer-bicarbonate buffer. Isometric contractions were measured with a force-displacement transducer and polygraph as described previously (Thomas et al., 1993). The ileum was allowed to incubate for 1 h, and then three test doses of the muscarinic agonist oxotremorine-M were added to ensure that the contractions were reproducible and of sufficient magnitude. The ileum was washed and allowed to rest for 5 min between each test dose. The longitudinal fundic strip, zig-zag tracheal strip, and isolated colon (proximal and distal) were prepared as described previously (Thomas and Ehlert, 1996; Sawyer and Ehlert, 1998). Aortas and femoral arteries were dissected from adult rats and mounted in organ baths (10 ml) using procedures similar to those described above. Tension was recorded on a Grass polygraph with initial preloads of 0.5 g for intestinal and tracheal preparations and 2 g for aorta and femoral artery.

Iodination. Prokineticin 1 was iodinated by the iodogen method as described previously (Fraker and Speck, 1978). Briefly, refolded prokineticin 1 (7.5 μg) was incubated with 50 μg of iodogen in 50 μl of 0.5 M PBS, pH 7.2, for 15 min at room temperature. The reaction was stopped by transferring the mixture to a Microfuge tube containing 100 μl of PBS, 1 mM NaI, and 0.1% bovine serum albumin. Free iodine was removed by gel filtration on Bio-Gel P2, and the radioactivity in the void volume was measured. Assuming that all the radioactivity had been incorporated into 6.0 μg of prokineticin 1 (80% recovery rate), we calculate that the specific radioactivity as 372 Ci/mmol.

Receptor Binding. Membranes were prepared from guinea pig ileum as described (Li et al., 2000), except additional steps of differential centrifugation (800 g, 10,000 g, 100,000 g, 4°C, 20 min each) were applied to reduce the background binding. Incubation was performed in 4 ml of 20 mM Tris-HCl buffer, pH 7.4, containing 0.1% bovine serum albumin at room temperature. For saturation binding, 1.5 to 200 pM labeled prokineticin 1 was used. Nonspecific binding was defined in the presence of 20 nM unlabeled prokineticin 1. For displacement experiments, unlabeled protein was preincubated with membrane in 3 ml of total reaction volume for 1 h, then ^{125}I-prokineticin 1 (20 pM) was added. The membrane was incubated for an additional 3 h at room temperature. The binding mixture was filtered through GF-C glass filters and washed with 10 ml of 20 mM Tris-HCl, pH 7.4. Radioactivity retained on filters was measured in a gamma counter. The data were analyzed with the LIGAND program.

Results

Identification and Analysis of Two Mammalian Homologs for Frog Bv8 and Snake MIT1. In an effort to identify mammalian homologs of frog Bv8 and snake MIT1, we searched multiple databases using the BLAST 2.1 algorithm (Altschul et al., 1997), with their protein sequences as queries. A search of the genome survey sequence and the high throughput genome sequence databases revealed a number of human bacterial artificial chromosome clones containing open reading frames homologous to Bv8 and MIT1. A further search of the EST database using the predicted human coding and 3′untranslated regions revealed the presence of two human EST sequences (ai277349 and aa883760). Sequence analysis revealed that aa883760 encodes a predicted protein (Heijne, 1986) with a signal peptide of 19 amino acids and a mature protein of 86 amino acids. Clone ai277349 was found to be a partial cDNA. Full-length sequence for EST clone ai277349, cloned by 5′ RACE with human brain cDNA as a template, was found to contain a signal peptide of 27 amino acids and a mature protein of 81 amino acids (Fig. 1). These proteins were named prokineticin 1 and prokineticin 2 (GenBank accession numbers AF333024 and AF333025), respectively (see below).

Sequence analysis reveals that prokineticins 1 and 2 con-

tain all 10 conserved cysteines and have about 44% amino acid identity. Both prokineticins possess about 43% identity with frog Bv8 and snake MIT1. Interestingly, the N-terminal sequences before the first cysteine (AVITGA) is completely conserved among all species (Fig. 1), suggesting a functional role for this region. Preliminary analysis of the mouse prokineticin 1 gene (data not shown) indicates that the N-terminal sequence AVITG is derived from the first exon that also contains the signal peptide sequence, whereas the cysteine-rich sequences are from other exon(s).

Prokineticins Are Expressed in GI and Other Tissues. As an initial survey of prokineticin expression, we probed a human master blot using fragments of human prokineticin cDNAs. Both prokineticins were widely expressed in various adult and fetal tissues, with a generally higher expression level of prokineticin 1 compared with prokineticin 2 (Fig. 2, A and B). The expression of prokineticins in peripheral tissues was further examined by Northern blot analysis. Figure 2D showed that prokineticin 1 is highly expressed in GI tissues, with the highest expression level in stomach, whereas prokineticin 2 expression can only be detected at low levels in small intestine. Interestingly, there are two different sizes of prokineticin 1 mRNA (1.5 and 1.8 kilobase pairs) among tissues examined, suggesting alternative splicing or alternative polyadenylation mechanisms for prokineticin 1.

Production, Refolding, and Purification of Human Prokineticins. Because the N-terminal sequences were completely conserved (Fig. 1), recombinant proteins with authentic N-terminal residue were produced first as GST-fusion protein, followed by the digestion with protease factor Xa to remove the GST tag. Figure 3 shows that a protein with correct molecular mass was produced by factor Xa digestion. Bioassay with the guinea pig ileum showed that the unfolded recombinant proteins were inactive (data not shown). As NMR examination indicated that the 10 cysteines of MIT1 are formed into five disulfide bonds (Boisbouvier et al., 1998) and that these 10 cysteines are all conserved in human prokineticin cDNAs, it seems likely that these disulfide bonds are probably essential for bioactivity. Thus, considerable effort was directed toward the attainment of the proper

```
                                          ↓
A)          MRGATRVSIMLLLVTVSDC  AVITGA
B) MRSLCCAPLLLLLLLPPLLLTPRAGDA  AVITGA
C)          MKCFAQIVVLLLVIAFSHG  AVITGA
D)                               AVITGA

CERDVQCGAGTCCAISLWLRGLRMCTPLGREGEECHPG
CDKDSQCGGGMCCAVSIWVKSIRICTPMGKLGDSCHPL
CDKDVQCGSGTCCAASAWSRNIRFCIPLGNSGEDCHPA
CERDLQCGKGTCCAVSLWIKSVRVCTPVGTSGEDCHPA
*     *    **          *       *

SHKVPFFRKRKHHTCPCLPNLLCSRFPDGRYRCSMDLKNINF
TRKVPFFGRRMHHTCPCLPGLACLRTSFNRFICLAQK
SHKVPYDGKRLSSLCPCKSGLTCSK.SGEKFKCS
SHKIPFSGQRMHHTCPCAPNLACVQTSPKKFKCLSKS
              * *     *       *
```

Fig. 1. Amino acid sequences of prokineticin 1 (A), prokineticin 2 (B), Frog Bv8 (C), and partial sequence of MIT 1 (D). Ten conservative cysteine residues are marked (*). Signal peptides are underlined. Arrow indicates the splice sites for introns.

configuration of disulfide bonds (of 945 possible combinations). Initial refolding after a single dilution in refolding buffer was unsuccessful. Almost all of the recombinant proteins precipitated, probably because of the formation of intermolecular disulfide bonds. A series of modifications to control protein aggregation and to slow disulfide bond formation were then adopted (Georgiou and Valax, 1996; Lilie et al., 1998). These modifications included: 1) the reduction in the protein concentration to 100 μg/ml or less to favor the formation of intra- but not intermolecular disulfide bonds; 2) the use of dialysis instead of direct dilution; 3) the use of higher levels of urea (4 M and then 2 M) in all dialysis buffers; 4) the omission of the oxidants cystine or oxidized glutathione from redox pairs, leaving only 5 mM cysteine or 3 mM reduced glutathione; 5) the addition of glycerol to further reduce protein aggregation; and 6) the cooling of proteins and buffers to 4°C before initiating the refolding process. These carefully controlled steps allowed us to refold recombinant prokineticins successfully with minimal protein aggregation.

The refolded proteins were finally purified by RP-HPLC (Fig. 3, A, lane 5, and B). Mass spectrometry confirmed the formation of five disulfide bonds in refolded recombinant prokineticin 1. The molecular mass of 6xHis-tagged prokineticin 1, determined with a Fourier transform mass spectrometer, was found to be 10480.30 Da (Fig. 3C). Because the calculated molecular mass with all 10 cysteines present in reduced form was 10490.20, five pairs of disulfide bonds were clearly formed.

Refolded Recombinant Prokineticins Potently Contract Gastrointestinal Smooth Muscle. The refolded recombinant prokineticins were tested on isolated smooth muscle preparations. Figure 4 shows that both recombinant prokineticin 1 and prokineticin 2 potently stimulated the contraction of guinea pig ileum longitudinal muscle with EC_{50} values of about 0.46 and 0.90 nM, respectively. Prokineticin 1 (5 nM) also stimulated contraction of the fundic muscle strip and proximal colon, but had no effect on distal colon (25 nM, data not shown). Recombinant prokineticin 1 (25 nM) also had no effect on other smooth muscle tissues, including aorta, femoral artery, trachea, and gallbladder (data not shown). Thus, among the tissue tested, the contractile effect of prokineticins seems specific for GI smooth muscle.

To investigate the possible signaling mechanisms of prokineticins, a number of kinase and ion channel inhibitors were tested. Tetrodotoxin, which is known to block nerve action potential propagation, had no effect on prokineticin 1-stimulated contractions of the longitudinal muscle of the ileum (Fig. 4B), indicating that prokineticin 1 acts directly on the smooth muscle. A number of compounds including the protein kinase C inhibitor calphostin C (1 μM), the phospholipase A2 inhibitor 7,7-dimethyl-(5*Z*,8*Z*)-eicosadienoic acid (10 μM), the tyrosine kinase inhibitor genistein (5 μM), the mitogen-activated protein kinase kinase inhibitor PD 098059 (10 μM), and the L-type calcium channel blockers verapamil and nifedipine were also investigated for their effects on prokineticin 1-induced contraction. Only the L-type calcium channel blockers were effective. At 100 nM, verapamil and nifedipine completely inhibited the contractile effect of 2 nM prokineticin 1 (Fig. 4C). The same concentration of verapamil and nifedipine also completely blocked the contractile action

of the muscarinic agonist oxotremorine-M (100 nM) (Fig. 4F). This result indicates that, like muscarinic M3 receptor-mediated contraction of the ileum (Eglen et al., 1996; Ehlert et al., 1997), calcium entry via the voltage-gated calcium channel is an essential component of prokineticin signaling.

Bioactivities of Prokineticins Are Mediated by Membrane Receptors. The potent contractile action of recombinant prokineticins on guinea pig GI smooth muscle and the inhibitory effect of the calcium channel blockers suggest a receptor-mediated mechanism for prokineticins. To provide direct evidence that prokineticins are interacting with selective membrane receptors, we labeled recombinant prokineticin 1 with ^{125}I and carried out receptor binding experiments. Prokineticin 1 saturably labeled guinea pig ileum with high affinity. Scatchard analysis indicated that the specific binding of prokineticin 1 was best fitted with two-site model (F = 38.78, $P < 0.001$ verse one site model; Fig. 5A). The high- and low-affinity constants (K_d) were 5.0 ± 0.8 pM and 227 ± 63 pM (n = 3), respectively. The B_{max} for high- and low-affinity sites were 7.8 ± 1.2 and 26.4 ± 8.4 fmol/mg of protein, respectively (n = 3). Competition experiments revealed that the specific binding was displaced by recombinant prokineticin 1. The displacement curves were also best fitted with two-site model (with K_i of 8.0 ± 3.9 pM, and 1.50 ± 0.9 nM, n = 3 for high- and low-affinity sites, respectively) (Fig. 5B). Figure 5B also showed that prokineticin 2 displaced labeled prokineticin 1 with similar affinity (K_i of 4.2 pM for high affinity and 1.22 nM for low affinity site, average of two experiments). Because agonist binding to many G protein-coupled receptors is inhibited by GTP, we investigated whether GTPγS had any effect on specific ^{125}I-labeled prokineticin 1 binding. Figure 5B shows that GTPγS caused a concentration-dependent inhibition of ^{125}I-prokineticin 1 binding. At the highest concentration tested (10 μM), GTPγS displaced 85% of the specific prokineticin binding to ileal membranes. These results suggest that prokineticin receptor(s) may belong to the G protein-coupled receptor family.

Discussion

Our results unequivocally established the existence of mammalian homologs of frog Bv8 and snake MIT1. To reflect their potent and specific effects on GI smooth muscle, we have named these proteins prokineticins. Their high potency in specifically stimulating the contraction of guinea pig ileal smooth muscle but not other smooth muscles including aorta, femoral artery, trachea, and gallbladder indicate that prokineticins may be important endogenous regulators of GI motility. Prokineticins may regulate GI smooth muscle as neurocrine-signaling molecules, circulating hormones, or paracrine humoral agents (Fox-Threlked, 1993; Burks, 1994; Wood, 1994). Because prokineticins are also widely expressed outside the GI tract, it is possible that prokineticins may be released from remote organs and regulate GI activity. In this respect, the resistance of prokineticins to protease treatment (unpublished observations) may guarantee their potential

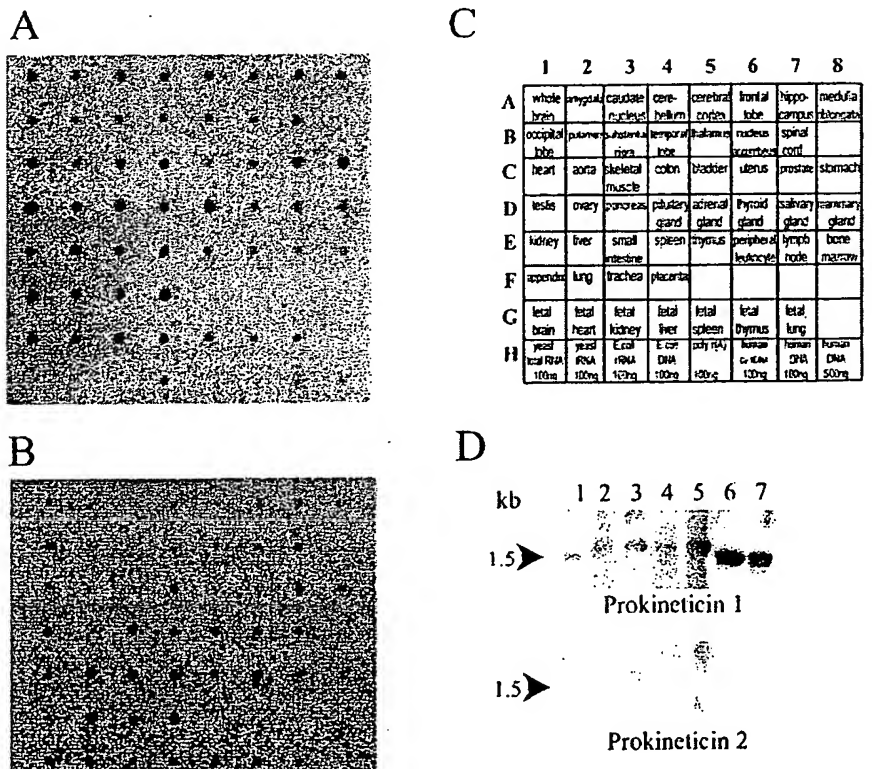

Fig. 2. Expression pattern of prokineticins. Human RNA master blot was probed with prokineticin 1 (A) and prokineticin 2 (B), respectively. C, diagram indicating the RNA sources for each dot. D, Northern blot analysis with prokineticin 1 and 2 in human peripheral tissues: 1, uterus; 2, colon; 3, small intestine; 4, bladder; 5, heart; 6, stomach; 7, prostate.

long-range and long-term effects. The molecular size and the processing of prokineticins distinguish them from typical neuropeptides, but render them more similar to cytokines (Loh et al., 1984; Vilcek, 1998). As one mechanism for eliminating pathogenic organisms is to enhance motility and push the offending organisms out of the GI tract, prokineticins may also be part of defending immune response (i.e., they function as inflammatory cytokines that increase the GI motility).

The high potency of recombinant prokineticins on the GI contractility suggests that prokineticins probably interact with cell surface receptor(s). This conclusion is reinforced by our receptor binding experiments, which demonstrate a saturable high affinity site for the iodinated recombinant prokineticin. Moreover, our observation that 10 μM GTPγS can displace almost all of the specific binding indicates the possible involvement of a G protein in prokineticin receptor signaling (Gilman, 1987; Gudermann et al., 1997). Moreover, the inhibitory effect of the calcium channel blockers verapamil and nifedipine on the contractile effect of prokineticin suggests a similar signaling mechanism for prokineticins and M3 muscarinic and motilin receptor in contracting GI smooth muscle: calcium entry via voltage-gated calcium channel is an essential component (Strunz et al., 1975; Eglen et al., 1996; Ehlert et al., 1997). Thus, the prokineticin receptor is likely to be a G protein-coupled receptor. However, other possibilities cannot be ruled out. For instance, prokineticins may cause smooth muscle contraction by directly activating

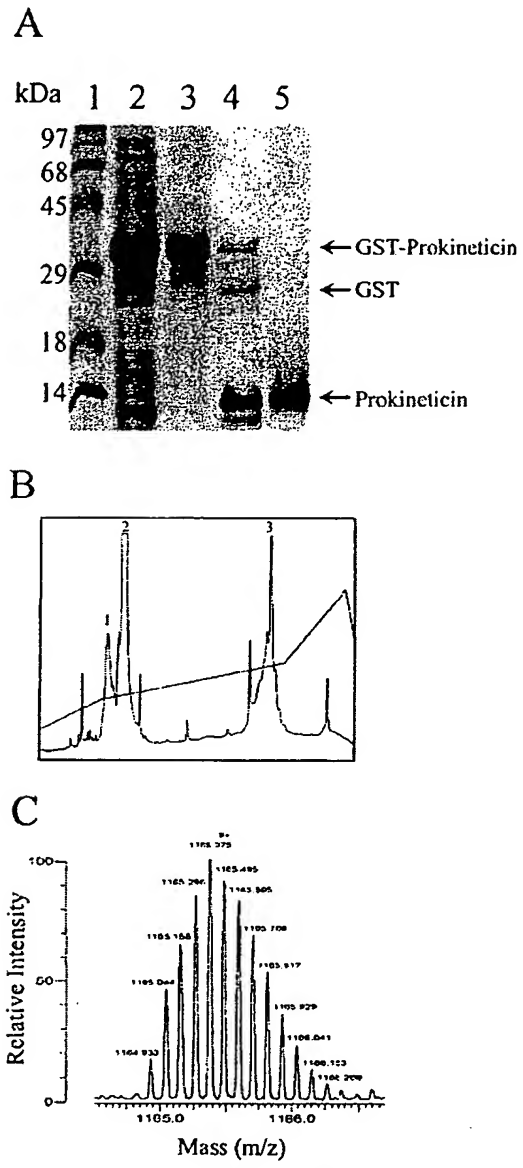

Fig. 3. Production and purification of human prokineticins. A, SDS-polyacrylamide gel electrophoresis (18%) of prokineticin 1 samples stained with Coomassie blue G-250. Lane 1, molecular mass standards; lane 2, whole cell lysate after induction; lane 3, Ni-NTA affinity chromatography-purified prokineticin; lane 4, factor Xa-digested prokineticin; lane 5, refolded prokineticin after HPLC purification. Each lane was loaded with 10 to 15 μg of total protein. B, reversed-phase HPLC separation of refolded protein mixture. Peak 2 contains refolded prokineticin. C, electrospray mass spectrum of refolded prokineticin 1 from peak 2 of RP-HPLC.

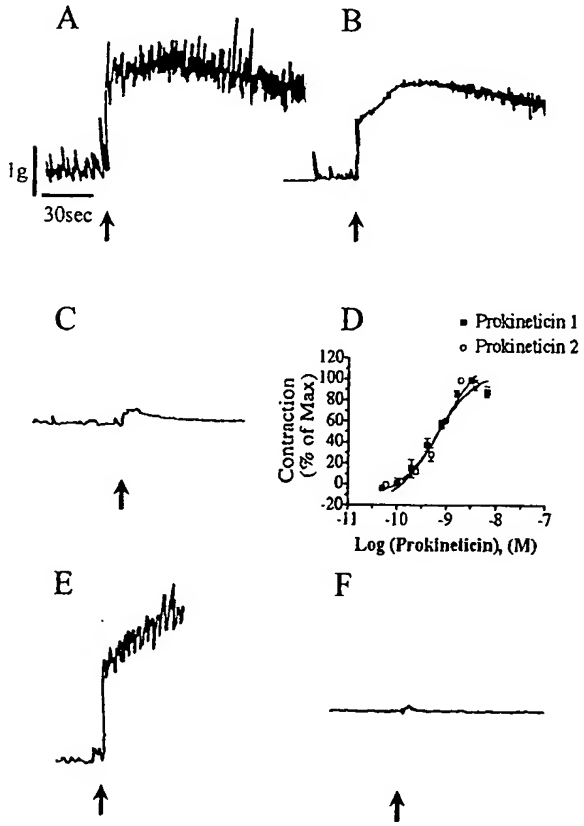

Fig. 4. Effects of prokineticins on the contractility of guinea pig ileal longitudinal muscle. The contractile responses to prokineticin 1 (2 nM) were measured in ileum in the absence (A) and in the presence of tetrodotoxin (0.1 μM; B) and verapamil (1 μM; C). D, concentration-response relationship for the contractile effects of prokineticins. Results are given as percentage of maximum contractility. Data are from three independent experiments. Contractile effects of oxotremorine-M in ileum in the absence (E) and in the presence of verapamil (1 μM; F) are also shown. Arrows indicate when drugs were added.

nonselective cation ion channels or by blocking inhibitory potassium channels on GI smooth muscle cells (Horowitz et al., 1996; Sanders, 1998). The availability of prokineticin receptor cDNA(s) should greatly facilitate the understanding of the prokineticin signaling mechanism.

Sequence analysis indicates that prokineticin may contain two functional domains—the short N terminus and the cysteine-rich C terminus. Because the N-terminal sequences preceding the first cysteine are completely conserved among prokineticins (Fig. 1), this region is likely to have functional importance. In addition to prokineticins and their isoforms from other species, a similar 10-cysteine motif is also found in a number of other secreted proteins including colipase, a cofactor for the intestinal lipid digestive enzyme lipase (van Tilbeurgh et al., 1992), and dickkopfs, a family of proteins that have an important role in early embryonic development (Aravind and Koonin, 1998; Glinka et al., 1998). Interestingly, dickkopfs actually possess two groups of 10-cysteine domains that have mirror symmetry. X-ray crystallography and solution structural analyses have clearly demonstrated that MIT1 has five pairs of disulfide bonds and is folded into a structure similar to colipase (Boisbouvier et al., 1998). Experiments with mutant and chimeric proteins should help to address the functional importance of prokineticin N-terminal and C-terminal domains. Wechselberger et al. (1999) have recently reported a mammalian cDNA sequence corresponding to prokineticin 2 here, but no functional studies were carried out. Interestingly, the cDNA sequence they reported has an insertion that encodes an extra 21 amino acids, suggesting the existence of alternative spliced form of prokineticin 2 in the testis. The functional significance of this alternative spliced form remains unclear.

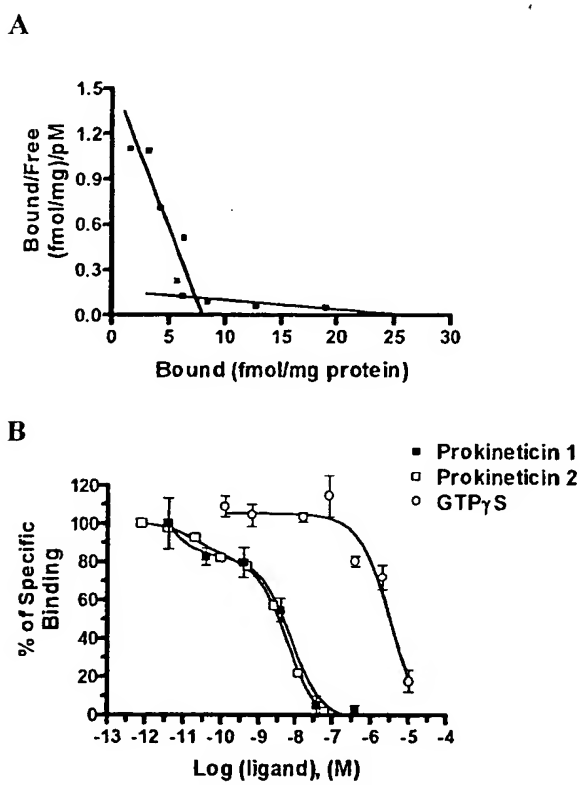

Fig. 5. A, Scatchard analysis of the specific binding of ^{125}I-prokineticin 1 to guinea pig ileal membranes. B, inhibitory effects of nonlabeled prokineticin 1 and GTPγS on the specific binding of ^{125}I-prokineticin 1 (20 pM) to a membrane preparation of the guinea pig ileum.

To our knowledge, this is the first report of proteins with five pairs of disulfide bonds that are successfully refolded in vitro. Refolding of proteins with more than three pairs of disulfide bonds is still regarded as challenging and difficult (Georgiou and Valax, 1996; Lilie et al., 1998). The expression of such disulfide bond-rich proteins in *E. coli* often results in a lack of formation of disulfide bonds or, more probably, the formation of incorrect intramolecular or intermolecular disulfide bonds. These events routinely lead to the production of inactive recombinant proteins and their aggregation in bacterial inclusion bodies. In this study, we used a slow exchange method to refold prokineticins that contain five pairs of disulfide bonds. A number of factors eventually contributed to our successful refolding of prokineticins: 1) a slow rate of removal of denaturing agent; 2) the use of only reducing agents in the redox refolding mixture, thereby allowing the slow formation of disulfide bonds; 3) low temperature; 4) a high concentration of urea and glycerol in dialyzing buffer to prevent protein aggregation; 5) a low concentration of recombinant protein to favor the formation of intra- but not intermolecular disulfide bonds. These refolding conditions should be instrumental for the design of protocols for the refolding of other recombinant proteins possessing multiple disulfide bonds.

In summary, we have discovered two novel cDNAs encoding prokineticins. Refolded recombinant prokineticins potently and specifically stimulate the contraction of GI smooth muscle. Because impaired GI motility is a very common clinical manifestation in many disorders, including irritable bowel syndrome, diabetic gastroparesis, postoperational ileus, chronic constipation, and gastroesophageal reflux disease (Tonini, 1996; Samsom and Smout, 1997; Achem and Robinson, 1998; Briejer et al., 1999), the discovery of an endogenous regulator of GI smooth muscle should facilitate the development of novel therapeutics for such disorders.

Acknowledgments

We thank Dr. R. Purdy and C. Kahwaji for performing the aorta experiment, Dr. R. McIver and G. Xu for mass spectrometer study, J. Bermak for graphic work, and Dr. D. Piomelli and P. Weingarten for critical comments on the manuscript. C.B. is currently a member of UC Irvine medical scientist training program.

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